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Investigation of the induction of DNA double-strand breaks by methylenediphenyl-4,4'-diisocyanate (MDI) in cultured human epithelial lung cells

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Investigation of the induction of DNA double-strand breaks by methylenediphenyl-4,4'-diisocyanate (MDI) in cultured human epithelial lung cells

FINAL REPORT

by

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Investigation of the induction of DNA double-strand breaks by methylenediphenyl-4,4'-diisocyanate (MDI) in cultured human lung epithelial cells

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Running Head: MDI and DNA double-strand breaks

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Investigation of th induction of DNA double-strand breaks by methylenediphenyl-4,4'-diisocyanate (MDI) in cultured human lung epithelial cells. Vock, E.H., Vamvakas, S., Gahlmann, R., Lutz, W.K. (1998). *Toxicological Sciences* ....

The question was addressed whether methylenediphenyl-4,4'-diisocyanate (MDI), a bifunctional electrophile, can induce DNA double-strand breaks (DSB) by interstrand DNA crosslink formation or whether DSB are the result of cell death. Cultured human lung epithelial cells (A549) were treated with MDI, methylene-4,4'-dianiline (MDA; a potential hydrolysis product of MDI), the nitrogen mustard melphalan and the detergent Triton X-100. All chemicals were dissolved in ethylene glycol dimethyl ether which was added to a cell monolayer covered with phosphate-buffered saline. After 2 hours, the treatment solution was exchanged against medium, and 8, 24 and 72 h after treatment initiation, the induction of DNA double-strand breaks was assessed by pulsed-field gel electrophoresis. At the same time, the viability was determined with the MTT test (intracellular reduction of the tetrazolium dye MTT). At the 8-hour time point, 1 and 10 µM melphalan induced DSB without concomitant effect on cell viability. With all other chemicals, the dose-response curves for DNA fragmentation and viability were mirror images. Approximate 50 % lethal concentrations were 200, 3000, and 100 µM for MDI, MDA, and Triton X-100, respectively. For these chemicals, the observed DSB were the consequence of extragenomic damage in the course of cell death rather than of an interaction with DNA. The mechanistic difference to melphalan was supported by the analysis of nuclear morphology. Apoptotic bodies were observed only after melphalan treatment whereas MDI and Triton X-100 only produced irregular clumping of chromatin (72-hour time point). DNA fragment length analysis showed a time-independent pattern with sizes between 1 and 4 Mbp for melphalan, while MDI, and Triton X-100 induced smaller DNA fragments in a time-dependent manner. It is concluded that DSB observed in cells treated with MDI are unlikely to be the result of DNA crosslink formation.

Introduction

Methylenediphenyl-4,4'-diisocyanate (MDI) is a bifunctional monomer widely used in the polyurethane production. Rats exposed by inhalation to an aerosol of technical MDI (6 hours per day, 5 days per week, for 24 months) developed adenomas (8/119) and one adenocarcinoma (1/119) in the lung at the highest dose level (6 mg/m^3) (Reuzel *et al.*, 1994). For a risk assessment at low dose, it is important to investigate whether the observed changes were due to a genotoxic mechanism, e.g. from covalent binding to DNA, or to a non-genotoxic mode of action, e.g., by cytotoxicity and regenerative processes.

DNA-adduct formation by MDI had been shown with ^{32}P-postlabeling in the epidermis of topically treated rats (Vock *et al.*, 1995). However, no such DNA adducts were detectable in the lung of rats exposed to a pure MDI aerosol for 1 year (Vock *et al.*, 1996). As a bifunctional electrophile, MDI has the potential to form DNA-DNA crosslinks which could result in DNA double-strand breaks (DSB) upon repair. The ^{32}P-postlabeling assay would not have detected this type of DNA damage.

MDI has been reported to induce chromosome aberrations in cultures of human lymphocytes (Maki-Paakkanen and Norppa, 1987) and in Chinese hamster fibroblasts (Ishidate, 1988). In addition, detection of double-strand breaks in DNA isolated from white blood cells of a worker after inhalation exposure to MDI had been reported (Marczynski *et al.*, 1992). However, the methods used in the latter study – polyacrylamide gel electrophoresis and anion exchange chromatography – are not sufficiently validated for the mechanistic interpretation of DNA double-strand break formation.

DSB may be formed by two pathways, (i) in the course of repair of interstrand DNA-DNA crosslinks or (ii) indirectly, in the course of cell death, by the action of nucleases. By comparing the dose-response curves for cell viability and for the formation of double stranded DNA fragments these two pathogenetic modes of DSB formation can be discriminated. Genotoxic chemicals are expected to induce DSB

with concentrations that do not induce cell death (Fig. 1, left). In contrast, the curve for DSB induced primarily via nongenotoxic mechanisms is expected to be a mirror image of the viability curve (Fig. 1, right).

In the present study, pulsed-field gel electrophoresis (PFGE) was used to investigate the dose- and time dependence of the formation of DSB in cultured human lung epithelial cells (A549) treated with MDI. For comparison, the aromatic amine methylene-4,4'-dianiline (MDA; the product obtained by hydrolysis and decarboxylation of both isocyanate groups of MDI), the bifunctional nitrogen mustard melphalan (Osborne and Lawley, 1993; Osborne *et al.*, 1995); and the detergent Triton X-100 were used.

Materials and methods

Materials

Cells: A549, a human epithelial like lung carcinoma cell line, was purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, USA.

Chemicals: Methylenediphenyl-4,4'-diisocyanate (Lupranat MR) was provided by BASF AG, Ludwigshafen, Germany. Methylenedianiline (MDA), melphalan, Triton X-100, Trypsin-EDTA, (3-[4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Hoechst bisbenzimide No. 33258, tricaprylin, corn oil and 1,2-dimethoxyethane (= ethylene glycol dimethyl ether, EGDME) were purchased from Sigma-Aldrich-Fluka, Deisenhofen, Germany. [Methyl-^{3}H]thymidine ([^{3}H]Thd; 40 - 60 Ci/mmol) was from ICN Biomedicals GmbH, Meckenheim, Germany. Cell culture flasks were from Greiner Labortechnik, Frickenhausen, Germany and microtiter plates from Nunc GmbH, Wiesbaden-Biebrich, Germany. The culture medium HamF12K and the foetal calf serum were supplied by Life Technologies, Eggenstein, Germany. Low melting point agarose and Gigaphore agarose were purchased from eurobio, Raunheim, Germany.

Cell culture and radiolabeling

A549 cells were grown as monolayer cultures at 37°C in humidified 5 % CO_2 atmosphere using HamF12K medium supplemented with 10 % foetal calf serum. For the PFGE experiments, approximately 400'000 cells from exponentially grown cultures were seeded in culture flasks (75 cm^2). Cells were labeled with 16.6 kBq/ml (0.45 µCi/ml) [^{3}H]Thd for 48 h, then washed with phosphate-buffered saline (PBS).

Treatments

MDI was liquefied at 60°C and a 1 M solution was freshly prepared in dry ethylene glycol dimethyl ether (EGDME; distilled over sodium and kept under

nitrogen), to minimize hydrolysis. Melphalan was dissolved in ethanol containing 0.4 % concentrated HCl (37 % w/v) and diluted 1:4 with EGDME. Stock solutions of MDA (1 M) and of Triton X-100 (5 %) were prepared in EDGME. After appropriate dilution, 400 µl of the solutions of the test chemicals were added to 9.6 ml PBS in the culture flasks held vertically, then within seconds the flask was laid horizontally and the liquid distributed over the cell monolayers. Control cells received the solvent only. After 2 hours, the PBS was replaced with complete medium and the cells were harvested after 8, 24 or 72 hours after treatment initiation. All experiments were repeated three times.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out according to standard procedures described in the literature (Blocher *et al.*, 1989; Cedervall *et al.*, 1995) with some modifications. The incubation flasks were placed on ice prior to harvest. Cells were detached with a rubber policeman, resuspended and collected in 15-ml tubes. After centrifugation, the cell pellet was washed once with ice-cold PBS/CMF (PBS/calcium-magnesium free). The cell number was determined by counting an aliquot of the cell suspension in a Coulter counter. The remaining cells were collected by centrifugation and mixed with 1 % low-melting-point (LMP) agarose in PBS at a concentration of 4 x 10^6 cells per ml. The mixture was immediately embedded into plug-former molds and chilled on ice for at least 30 minutes. The plugs were extruded, added to 2.5 ml lysis buffer (2 % *N*-lauryl-sarcosine, 0.5 M EDTA, 0.5 mg/ml proteinase K, pH 8) and incubated overnight at 50°C. Following lysis, the plugs were rinsed three times with 5 ml 10 mM Tris, 1 mM EDTA, pH 8 buffer (TE) and stored in the TE buffer at 4°C.

Agarose gels (0.8 %) were prepared in 40 mM Tris buffer (pH 8) containing 40 mM acetate and 1 : M EDTA (TAE buffer). Plugs were inserted into the wells using a hooked Pasteur pipette and sealed with 1 % LMP agarose. Chromosomal markers from *Schizosaccharomyces pombe* (*S.pombe*: 3.5, 4.68 and 5.7 Mbp) and *Saccharomyces cerevisiae* (*S.cerevisiae*: 0.24 up to 2.2 Mbp) were included in the

analysis. PFGE was performed using a BioRad CHEF-DR III system with block 1: 4.5 V/cm, a ramping of 3 to 5 seconds for 1 h with an angle of reorientation of 96°; followed by block 2: 2 V/cm with a constant pulse time of 1200 seconds for 24 h at 96° ; block 3: 2 V/cm, 1500 seconds, 24 h, 100°; block 4: 2 V/cm, 1800 seconds, 24 h, 106°. TAE buffer was circulated and cooled to 14°C throughout the electrophoresis. The agarose gels were stained for 30 minutes with 0.5 µg ethidium bromide/ml water, destained once in distilled water for 30 minutes and photographed using a UV transilluminator. For liquid scintillation counting the gel lanes were cut into 5 mm segments and placed in miniature glass vials. Each segment was dissolved in 0.25 ml 1 M HCl at 95°C, neutralised with 1 M NaOH, mixed with 7 ml Ultima Gold® (Packard) scintillation liquid and counted three times for 10 minutes in a TRI-CARB® 2250 CA liquid scintillation counter (Packard). Net dpm was determined by subtracting the cpm background values found in the lane of unlabeled cells and converted to dpm using the counting-efficiency.

DSB assessment: The fraction of DNA (activity) released (FAR) was calculated as follows:

FAR = dpm in the lane / (dpm in the lane + dpm in the plug) (Eq. 1)

Molecular size analysis: The data from the treated cells were corrected for background, i.e. the DNA in the lane obtained from the plugs with untreated cells. This correction assumes that in all treated samples an identical additional amount of DNA is released into the lane by the same degradative processes that released the DNA from control cells into the lane. Furthermore, the correction assumes that the migration of the additional DNA in the lanes of the treated cells will be the same as that observed for the controls.

The percentage of the total DNA (dpm) in a given segment after correcting for background (f_{corr}) is calculated by the following equation (Cedervall *et al.*, 1995):

$$f_{corr} = \frac{dpm_{t,si}}{dpm_{t,p} + dpm_{t,l}} - \frac{dpm_{c,si}}{dpm_{c,p} + dpm_{c,l}} \cdot \left(\frac{\frac{dpm_{t,p}}{dpm_{t,p} + dpm_{t,l}}}{\frac{dpm_{c,p}}{dpm_{c,p} + dpm_{c,l}}} \right) \quad \text{(Eq. 2)}$$

$dpm_{t,si}$ and $dpm_{c,si}$ is the radioactivity in a given segment i from treated and control cells, respectively, whereas $dpm_{t,p}$ and $dpm_{c,p}$ stands for the radioactivity in the plug and $dpm_{t,l}$ and $dpm_{c,l}$ in the lane from treated and control cells, respectively.

The calibration curve for molecular size as a function of migration distance was determined in the same gel with *S.pombe* and *S. cerevisiae* yeast standards. This curve was used to convert migration distance into molecular size, expressed in Mbp.

Cell survival assessed by the MTT test

One thousand cells per well were seeded in a 100 µl volume into 96-well plates. After incubation at 37°C, 5 % CO_2 for 3 days, cells were washed with PBS and treated in triplicate by adding 4 µl of the solutions of the test compounds to 96 µl PBS. After 2 hours, the PBS was replaced by medium and the test terminated after 8, 24 or 72 hours from beginning of treatment. Peripheral wells of each plate lacking cells were utilised for medium/tetrazolium reagent blank (n = 8) "background" (BG) determination and untreated cells (N=16) served as internal control. A 5 mg MTT/ml PBS stock solution was prepared, sterile filtered with a 0.2 µm polyamid filter (Schleicher & Schuell) and stored at 4°C for a maximum of 1 month. After the appropriate exposure time, the medium was removed and 100 µl medium containing 0.2 mg MTT/ml was added to each well, the culture plates were wrapped in aluminium foil and the cells incubated at 37°C for at least 4 hours (Borenfreund *et al.*, 1988). The medium was removed and replaced with 150 µl DMSO and 25 µl glycine buffer (0.1 M glycine, 0.1 M NaCl, adjusted to pH 10.5 with 1 M NaOH). The

absorbance (A) was recorded immediately at 570 nm using a MRX ELISA reader from Dynatech Laboratories. Results are means from 9 determinations obtained in three independent experiments. Cell survival was calculated as follows:

$$\text{Survival [\%]} = (A_{\text{treated}} - A_{BG}) * 100 / (A_{\text{solvent control}} - A_{BG}) \qquad \text{(Eq. 3)}$$

For comparison, the LC_{50} values of the test chemicals, i.e. the concentration inducing a 50 % reduction of cell viability were estimated.

Assessment of cell nucleus morphology

10'000 cells were seeded in 0.5 ml per well in 24 plates containing tissue culture cover slips, incubated for 2 days and treated with test chemicals concomitantly with the DSB assay. At the end of the treatment period, the cells were washed twice with PBS/CMF, fixed with ice-cold methanol and stored at -20°C. The fixed cells were stained with Hoechst bisbenzimide 33258 (5µg/ml PBS/CMF) for 2 minutes, washed twice with PBS/CMF and the coverslips placed invertedly on a slide. Nuclei were visualised with a fluorescence microscope. Apoptosis was defined by sharply circumscribed chromatin laid against the nuclear envelope and membrane blebs; necrosis was characterized by the irregular clumping of chromatin (Kerr *et al.*, 1995).

Results

Assay development

The present study in cultured A549 human lung epithelial cells aimed at discriminating between genotoxic and cytotoxic mechanisms of DSB induction by methylenediphenyl-4,4'-diisocyanate (MDI). Because of the strong electrophilic reactivity of the isocyanate groups, the first methodical task was to minimize hydrolysis and other reactions of MDI with nucleophiles before it reaches the cells. DMSO could not be used as solvent for MDI, because it had been reported to accelerate hydrolysis (Brown *et al.*, 1987; Gahlmann *et al.*, 1993). Tricaprylin and corn oil were too toxic to the cells. The best results were achieved with ethylene glycol dimethyl ether (EGDME) as solvent and addition of the solutions to PBS (not medium) in order to avoid all unnecessary nucleophiles able to react with the isocyanate groups. After an incubation period of 2 hours, PBS was replaced by medium and an expression period of 6, 22, and 70 hours was allowed. Under these conditions, the viability was 70 % as compared to untreated cells, most probably due to locally high concentrations of EDGME within the first seconds of mixing.

Correlation between DNA fragmentation and cell survival

The concentration of MDI required to reduce viability to 50 % of control (LC_{50}) was about 200 µM at all time points investigated. At the 8-hour time point, DSB were induced only at ≥100 µM and the FAR values increased with time (30, 60, and 70 % after 8, 24, and 72 hours, respectively). The dose-response curves for viability and FAR values showed a mirror image (Fig. 2), i.e., DNA double-strand break formation was observed only in connection with a reduction in cell viability. At 300 µM MDI and above, hydrolysis of the test chemical and formation of polyurea particles interfered with the assay.

The contribution of MDA, a potential hydrolysis product of MDI, to the effects seen with MDI was not substantial, since the LC_{50}-value of MDA ranged around 2-

3 mM, i.e. was more than 10-fold higher than MDI (Fig. 3). As with MDI, DSB induction was seen only at cytotoxic concentrations. These data can only be used as controls for the effects of MDI but do not provide full information on a putative crosslinking potential of MDA because the latter requires metabolic activation for the formation of reactive intermediates.

The bifunctional nitrogen mustard melphalan showed a clear dose-dependent increase of the FAR values in the absence of any impairment of cell viability, 8 hours after treatment initation (Fig. 4). Toxicity increased in a time-dependent manner with estimated LC_{50} values of 500, 100, and 5 µM, at 8, 24 and 72 hours, respectively. Melphalan at 10 µM induced DNA double strand breaks with a FAR of 90 % after 8 hours which remained at this level after 24 hours but decreased to 70 % after 72 hours. Melphalan at 1 µM showed a decrease of the FAR from 50 % after 8 hours to 30 and 10 % after 24 and 72 hours, respectively, without reduction of cell viability below 80 %. Hence the majority of the DSB initially induced by 1 µM melphalan were rejoined in the period between 8 and 72 hours.

The detergent Triton X-100 (Fig. 5) showed the a picture similar to the one seen with MDA or MDI. The LC_{50} value (at about 100 µM) remained unchanged over time. The dose-response curves for viability and DNA fragmentation were mirror images.

Fragment size distribution

Fig. 6 shows the molecular size distribution of the DNA fragments produced by MDI, Triton X-100 and melphalan after 8, 24, and 72 hours. Two distinct distribution patterns can be observed. MDI and the detergent Triton X-100 induced DNA fragments of a size of 2-3 Mbp after 8 hours (triangles). Later, the fragments became smaller (≤1 Mbp). The time-dependent decrease in fragment length could be explained by an indirect, enzymatic process. In contrast, most DNA fragments induced by melphalan were larger than 1 Mbp at all times and the size distribution was dependent on concentration more than on time.

Cell nucleus morphology

Apoptotic bodies were observed 72 hours after treatment with 10 µM melphalan. Treatment of the cells with 30 to 100 µM MDI or Triton X-100 only resulted in irregular clumping of chromatin. Hence, cell death induced by MDI appeared to be a necrotic rather than an apoptotic event.

Discussion

The investigation of the effects of isocyanates on cells is rendered difficult experimentally because of the strong reactivity of the functional groups. DMSO which is frequently used as solvent in bacterial (Andersen *et al.*, 1980; Shimuzu *et al.*, 1985) and mammalian cell test systems (Maki-Paakkanen and Norppa, 1987; Ishidate, 1988) even accelerates hydrolysis of MDI. In order to diminish loss of reactive isocyanate groups before the molecule reaches the cells, DMSO was replaced by EGDME, and PBS was used instead of medium to avoid constituen a carbohydrates and proteins which could also react with the isocyanate group. Even under these conditions probably only a fraction of the MDI dissolved in EGDME reached the cells intact. In addition, EGDME alone was also slightly toxic to the cells (30 % reduction in viability). Furthermore, the absence of nutrients and growth factors during the 2-hour exposure to the test chemical in EGDME/PBS appeared to affect some cellular functions, including those involved in the reduction of the MTT dye used to test for cell viability. An enzyme activation would be the most simple explanation for viabilities above 100 %, seen particularly at the 8-hour time point. Despite all this, triplicate determinations produced reproducible results for all endpoints so that the dose response of the effects of the test chemicals could well be investigated.

Viability, DNA double-strand break formation, and cell nucleus morphology were investigated at three different time points. At the 8-hour point, processes such as the uptake of the test chemicals by the cells, the reaction with target molecules, the formation of DSB as a consequence of the repair of DNA-DNA crosslinks, and the first stages of the process cell death are included. Enzyme induction and repair of DSB might come into play only at later time points, especially after the cells have passed through the cell cycle (about 48 hours for the A549 cells). Therefore, the investigation of the time dependence provided some information of the different processes involved. The 8-hour data allowed the clearest distinction whether the

compound tested induced strand breaks by a genotoxic or cytotoxic mode of action. The nitrogen mustard melphalan induced DSB by a genotoxic mechanism based on the fact that the FAR values increased up to 100 % without reduction in cell viability. In contrast, exposure to the detergent Triton X-100, MDI and MDA resulted in DSB formation only together with a reduction in cell survival. All three cytotoxic compounds had in common that the shape of the dose-response curves for viability did not change significantly between 8 and 72 hours whereas after treatment with the genotoxic melphalan the viability decreased in a time-dependent manner indicating that DSB also contributed to cell death.

Various mechanisms modulate the formation of DSB, e.g., the type of DNA damage, the DNA-repair capacity and whether programmed cell death or necrosis was induced (Vamvakas *et al.*, 1997). Therefore, at the 72 hour time point, the response to exogenous damage may show a larger variability than after 8 hours. For example, DSB increased between 8 and 24 h but decreased at 72 h for cells treated with Triton X-100, whereas no decrease was seen after exposure to MDI. At 30 µM MDI, the increase of the FAR value at 72 hours was significant, while the reduction in viability was not. The late process of DNA fragmentation could also be considered a consequence of cell death since it has been shown that lethally damaged cells can show normal metabolic activities for several days (Sorenson *et al.*, 1990). The fragment length data provided additional information and might also explain some of the above-mentioned time-dependent observations. For instance, an apparent decrease in DSB at 72 hours could be the result of such an extensive DNA degradation that small fragments moved out of the gel during electrophoresis.

The fragment length distribution corroborated the classification of MDI and Triton X-100 as cytotoxic as opposed to the genotoxic melphalan.

The aromatic di-amine MDA is formed by spontaneous hydrolysis and decarboxylation of both isocyanate groups of MDI, particularly under dilute conditions. (At higher concentrations of MDI, free amino groups react readily with remaining isocyanate groups, forming urea oligomers and polymers.) In order to

control whether an effect observed with MDI might have been due to the formation of MDA, this compound was included in our battery of test chemicals. In view of the finding that MDA was much less toxic than MDI and did not induce DSB by the genotoxic pathway, it can be concluded that the effects observed with MDI are the result of the isocyanate groups rather than of the amino groups. Whether MDA has a crosslinking potential on its own cannot be deduced from the present experiments because metabolic activation is required for the formation of the DNA-reactive intermediates. While some metabolic capacity of the A549 cells has been shown ([Wiebel and Singh, 1980), this activity would probably not reflect the situation *in vivo*. Data on DNA adduct formation of MDI (Vock and Lutz, 1997) and MDA (Schütze *et al*, 1996) in rat liver are available and indicate low potency only. Furthermore, the respective binding levels included mono-adducts which are expected to be much more frequent than crosslinks.

In conclusion, the data give no evidence that MDI induces DSB by a genotoxic mechanism based on its theoretical DNA-DNA crosslinking ability. It induced cell death by way of necrosis and not apoptosis. In rats, MDI induced tumors only concomitantly with histopathological signs of toxicity but in the absence of detectable DNA adducts in the target organ (Vock *et al.*, 1996). The available evidence indicates that tumor induction by MDI relies more on epigenetic modes of action resulting from cytotoxicity than on genotoxicity resulting form reactivity with DNA.

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Legends to Figures

Fig. 1: Schematic dose-response curves for the reduction of cell survival (——) and the induction of DNA double-strand breaks (---) as expected after treatment with a genotoxic (left) or cytotoxic (right) agent. _._._ symmetry axis.

Fig. 2. Dose-dependent induction of DNA double-strand breaks (---) and reduction of cell survival (——) analysed at different times after addition of methylenediphenyl-4,4'-diisocyanate (MDI) to monolayers of A549 cells. Results are means ± SD of 3 independent experiments.

Fig. 3. Dose-dependent induction of DNA double-strand breaks (---) and reduction of cell survival (——) analysed at different times after addition of methylene-4,4'-dianiline (MDA) to monolayers of A549 cells. Results are means ± SD of 3 independent experiments.

Fig. 4. Dose-dependent induction of DNA double-strand breaks (---) and reduction of cell survival (——) analysed at different times after addition of the nitrogen mustard melphalan to monolayers of A549 cells. Results are means ± SD of 3 independent experiments.

Fig. 5. Dose-dependent induction of DNA double-strand breaks (---) and reduction of cell survival (——) analysed at different time points after addition of the detergent Triton X-100 to monolayers of A549 cells. Results are means ± SD of 3 independent experiments.

Fig. 6. Molecular size distribution of DNA fragments induced by treatment of A549 cells for 2/8 hours (triangle), 2/24 hours (circle), and 2/72 hours (square), with 100 µM MDI, 80 µM Triton X-100, 1 and 10 µM melphalan.

Fig. 1

Cytotoxic mode of DSB-induction
Cell viability [—]
DNA double-strand breaks [---]
Concentration of test chemical
Genotoxic mode of DSB-induction
Cell viability [—]
DNA double-strand breaks [---]
Concentration of test chemical

Fig. 2

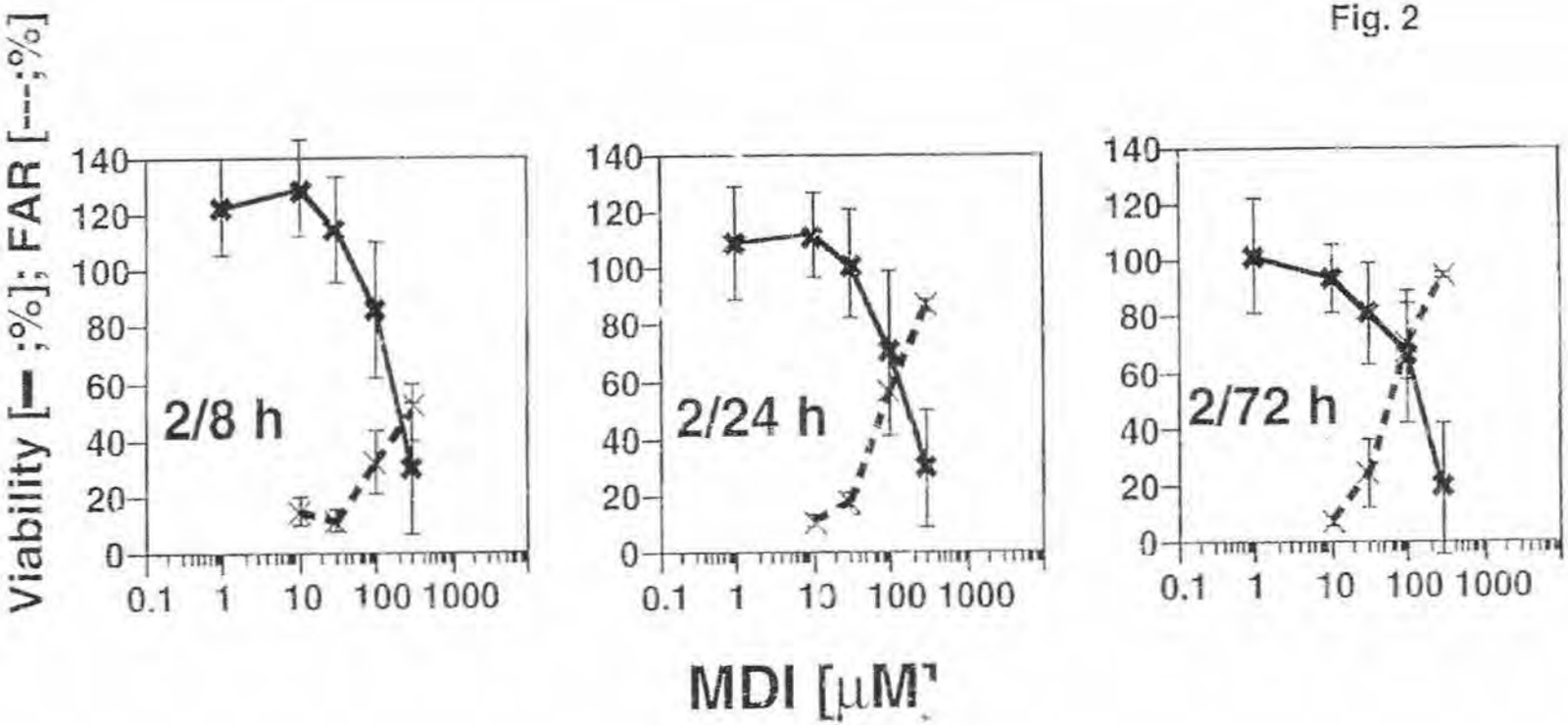

Viability [—;%]; FAR [---;%]
2/8 h
2/24 h
2/72 h
0
20
40
60
80
100
120
140
0.1
1
10
100
1000
MDI [µM]

Fig. 3

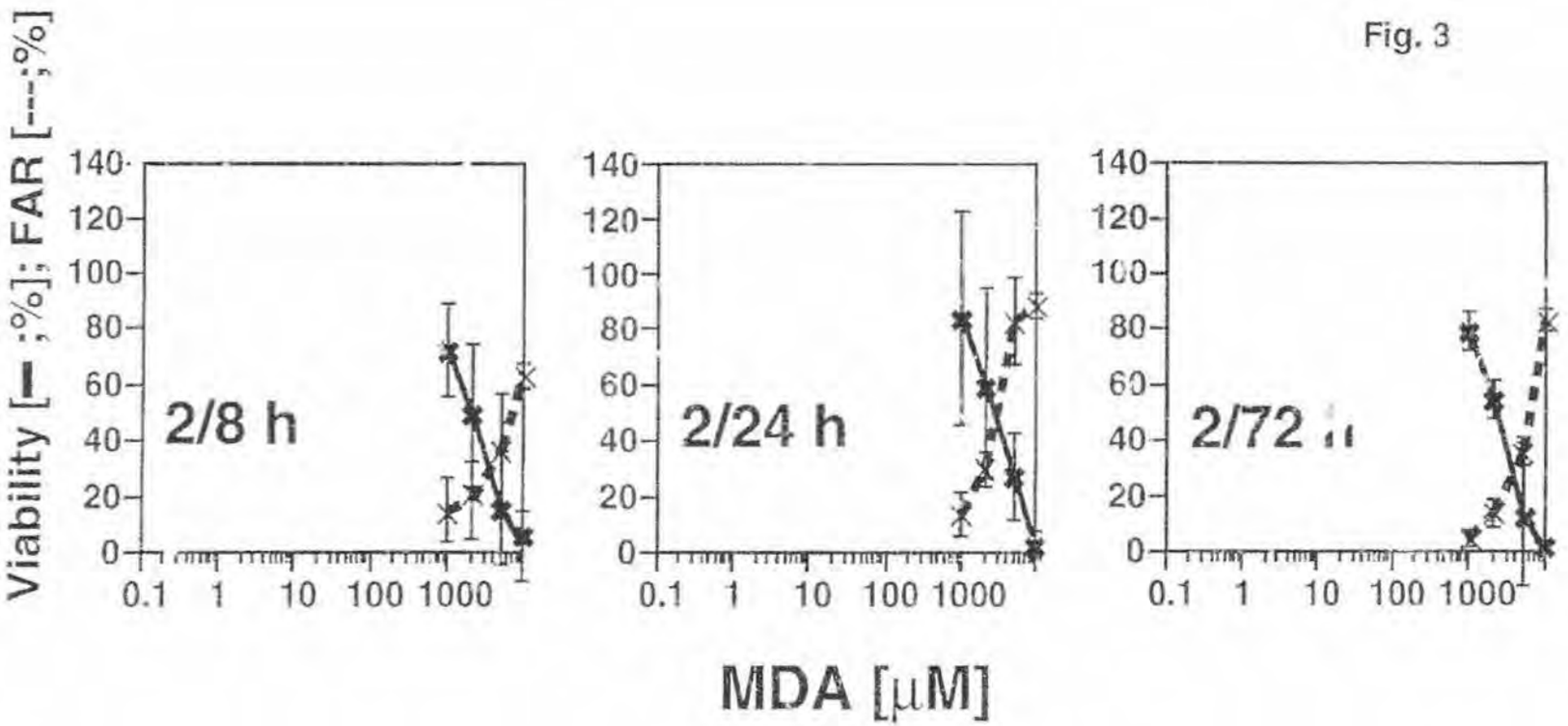

Viability [—;%]; FAR [---;%]
2/8 h
2/24 h
2/72 h
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
MDA [µM]

Fig. 4

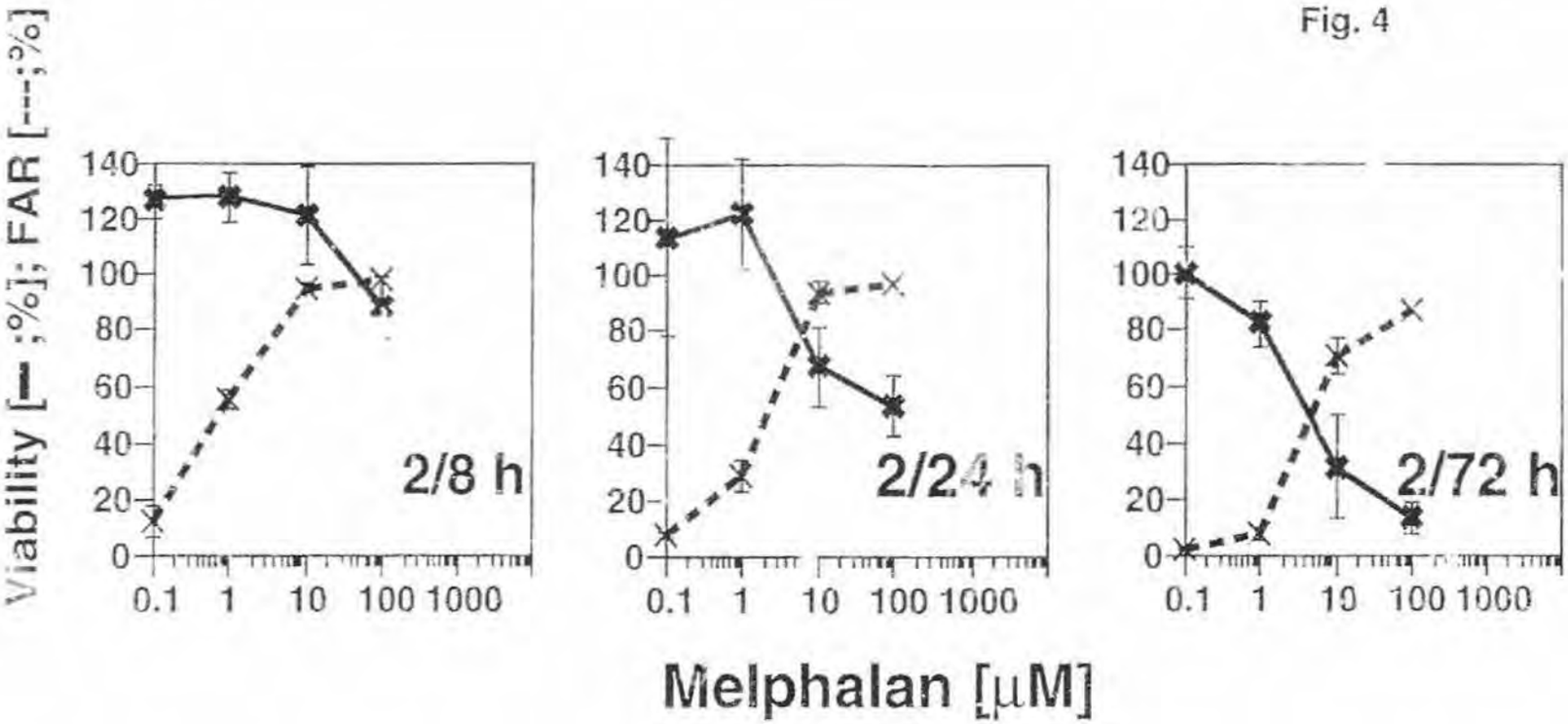
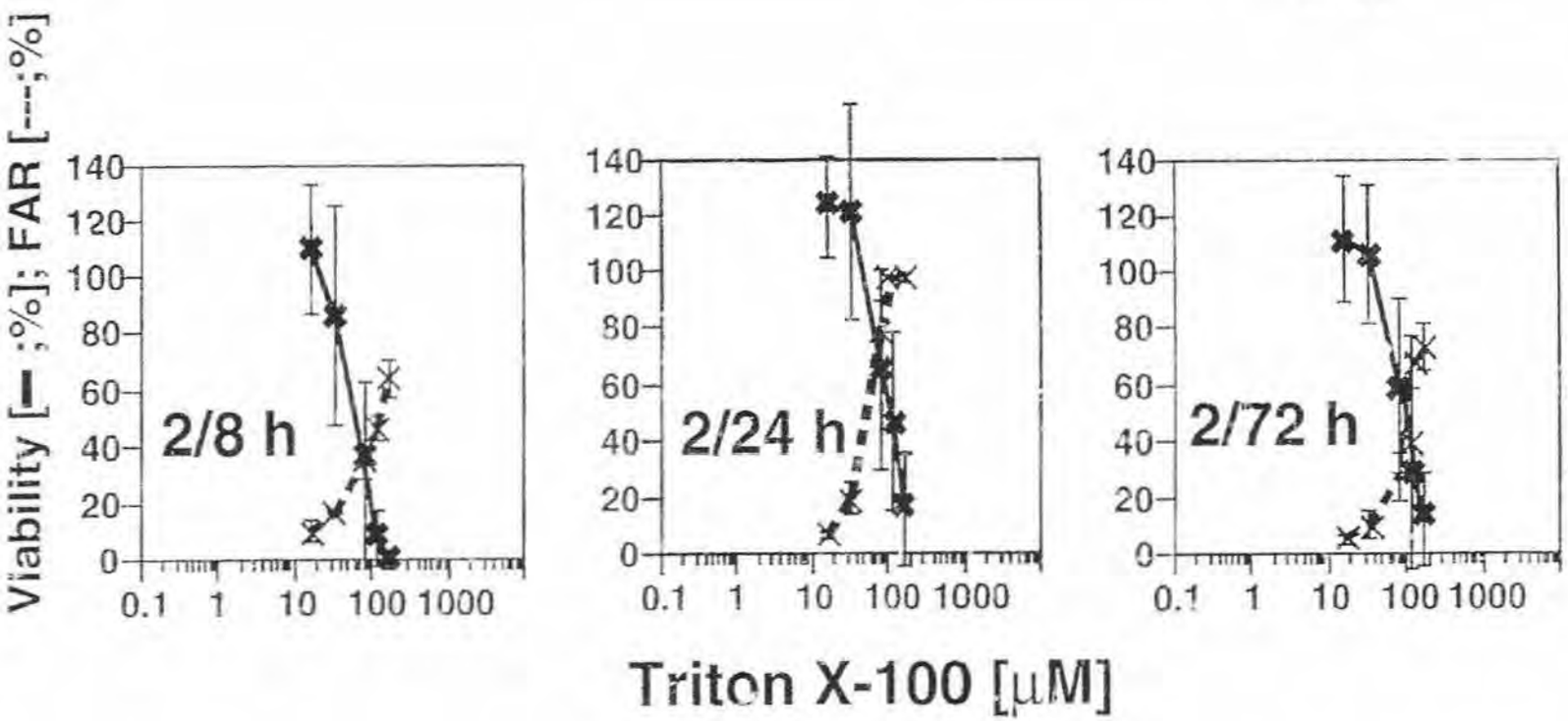
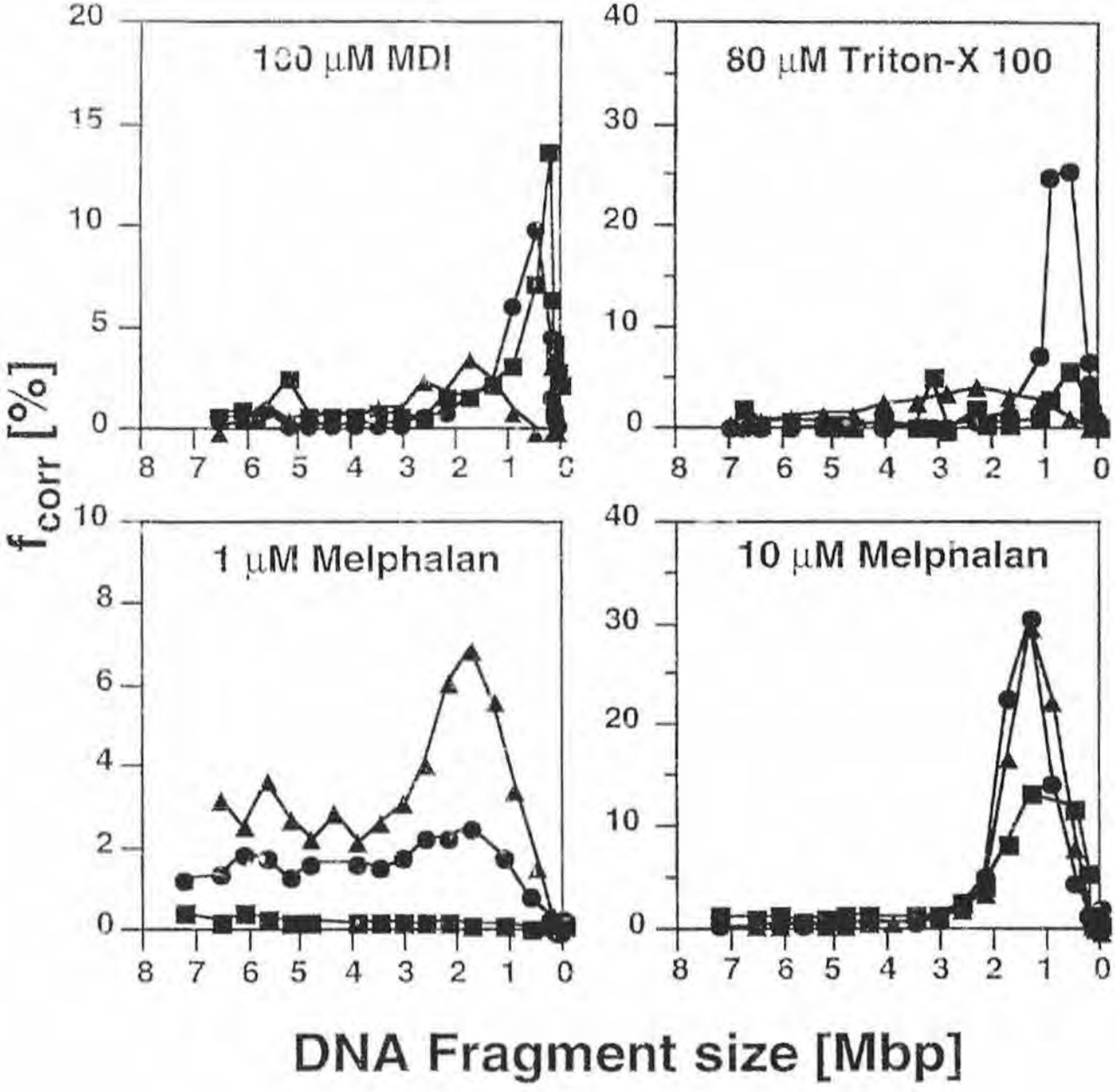

Viability [—;%]; FAR [---;%]
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
2/8 h
2/24 h
2/72 h
Melphalan [µM]

Fig. 5


Viability [—;%]; FAR [---;%]
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
2/8 h
2/24 h
2/72 h
Triton X-100 [µM]

C 03

Fig. 6


100 µM MDI
80 µM Triton-X 100
1 µM Melphalan
10 µM Melphalan
f corr [%]
DNA Fragment size [Mbp]

C 04

CERTIFICATE OF AUTHENTICITY

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